AN ISOLATED POLYPEPTIDE RELATED TO THE EPIDERMAL GROWTH FACTOR RECEPTOR, ANTIGEN THERETO, AND BIOASSAYS AND METHODS RELATED THERETO

This application is a continuation-in-part of applicant's pending U.S. Serial No. 07/444,406, which was filed on December 4, 1989.

FIELD OF THE INVENTION

The present invention relates to genes which encode novel proteins related to a family of receptor proteins typified by two related membrane spanning tyrosine kinases: the Epidermal Growth Factor receptor (EGFR), which is encoded by the *erb*B gene, the normal human counterpart of an oncogene (*v-erb*B) that was first recognized in the proviral DNA of avian erythroblastosis virus; and the receptor encoded by the related gene *erb*B-2. In particular, the present invention relates to a DNA segment encoding the coding sequence, or a unique portion thereof, for a third member of this receptor gene family, herein designated *erb*B-3.

BACKGROUND OF THE INVENTION

Proto-oncogenes encoding growth factor receptors constitute several distinct families with close overall structural homology. The highest degree of homology is observed in their catalytic domains, essential for the intrinsic tyrosine kinase activity of these proteins. Examples of such receptor families include: the EGFR and the related product of the *erb*B-2 oncogene; the Colony Stimulating Factor 1 receptor (CSF-1-R) and the related Platelet-Derived Growth Factor receptor (PDGF-R); the insulin receptor (IR) and the related Insulin-like Growth factor 1 receptor (IGF-1R); and the receptors encoded by the related oncogenes *eph* and *elk*.

It is well established that growth factor receptors in several of these families play critical roles in regulation of normal growth and development. Recent studies in *Drosophila* have emphasized how critical and multifunctional are developmental processes mediated by ligand-receptor interactions. An increasing number of *Drosophila* mutants with often varying phenotypes have now been identified as being due to lesions in genes encoding such proteins. The genetic locus of the *Drosophila* EGFR homologue, designated *DER*, has recently been identified as being allelic to the zygotic embryonic lethal *faint little ball* exhibiting a complex phenotype with deterioration of multiple tissue components of ectodermal origin. Furthermore, other mutants appear to lack *DER* function either in the egg or the surrounding maternal tissue. Thus, the *DER* receptor may play an important role in the ligand-receptor interaction between egg and follicle cells necessary for determination of correct shape of eggshell and embryo. It is not yet known whether *DER* represents the sole *Drosophila* counterpart of known mammalian *erb*B-related genes.

Some of these receptor molecules have been implicated in the neoplastic process as well. In particular, both the *erb*B and *erb*B-2 genes have been shown to be activated as oncogenes by mechanisms involving overexpression or mutations that constitutively activate the catalytic activity of their encoded receptor proteins (Bargmann, C. I., Hung, M. C. & Weinberg, R. A., 1986, *Cell* 45:649−657; Di Fiore, P. P., Pierce, J. H., Kraus, M. H., Segatto, O., King, C. R. & Aaronson, S. A., 1987, *Science* 237:178−182; Di Fiore, P. P., Pierce, J. H., Fleming, T. P., Hazan, R., Ullrich, A., King, C. R., Schlessinger, J. & Aaronson, S. A., 1987, *Cell* 51:1063−1070; Velu, T. J., Beguinot, L., Vass, W. C., Willingham, M. C., Merlino, G. T., Pastan, I. & Lowy, D.R., 1987, *Science* 238:1408−1410). Both *erb*B and *erb*B-2 have been causally implicated in human malignancy. *erb*B gene amplification or overexpression, or a combination of both, has been demonstrated in squamous cell carcinomas and glioblastomas (Libermann, T. A., Nusbaum, H. R., Razon, N., Kris, R., Lax, I., Soreq, H., Whittle, N., Waterfield, M. D., Ullrich, A. & Schlessinger, J., 1985, *Nature*

313:144–147). *erb*B-2 amplification and overexpression have been observed in human breast and ovarian carcinomas (King, C. R., Kraus, M. H. & Aaronson, S. A., 1985, *Science* 229:974–976; Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., Ullrich, A. & Press, M. F., 1989, *Science* 244:707–712), and *erb*B-2 overexpression has been reported to be an important prognostic indicator of particularly aggressive tumors (Slamon, D. J., *et al.*, 1989, *supra*). Yet, not all such tumors have been found to overexpress *erb*B-2, and many human tumors have not yet been associated with any known oncogene. Thus, there has been a continuing need to search for additional oncogenes which would provide knowledge and methods for diagnosis and, ultimately, for rational molecular therapy of human cancers.

Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

SUMMARY OF THE INVENTION

It is an object of present invention to provide a DNA segment encoding a receptor protein related to the *erb*B proto-oncogene family which previously has not been known or even suspected to exist. Further, it is an object of the present invention to develop assays for expression of the RNA and protein products of such genes to enable determining whether abnormal expression of such genes is involved in human cancers. Thus, further objects of this invention include providing antibodies, either polyclonal or monoclonal, specific to a unique portion of the receptor protein; a method for detecting the presence of an *erb*B-3 ligand that is capable of either activating or down-regulating the receptor protein as well as procedures for purifying the resultant ligand; a method of screening potential ligand analogs for their ability to activate

the receptor protein; and procedures for targeting a therapeutic drug to cells having a high level of the receptor protein.

In pursuit of the above objects, the present inventors have discovered a human genomic DNA fragment that is produced by cleavage with the *Sac*I restriction enzyme, has a size of about 9 kbp, and is detectable by nucleic acid hybridization with a probe derived from the *v-erb*B gene only under reduced stringency hybridization conditions. Thus, this DNA fragment is distinct from those known to encode the epidermal growth factor receptor (EGFR) (i.e., the *erb*B gene) and from the related *erb*B-2 gene. Characterization of this DNA fragment after partial purification and molecular cloning showed that the region of *v-erb*B homology mapped to three exons that encode amino acid sequences having homologies of 64% and 67% to contiguous regions within the tyrosine kinase domains of the EGFR and *erb*B-2 proteins, respectively. A probe derived from the genomic DNA clone identified cDNA clones of the related mRNA which encode a predicted 148 kd transmembrane polypeptide with structural features identifying it as a member of the *erb*B family, prompting designation of the new gene as *erb*B-3. This gene was mapped to human chromosome 12q11−13 and was shown to be expressed as a 6.2 kb transcript in a variety of normal tissues of epithelial origin. Markedly elevated *erb*B-3 mRNA levels were demonstrated in certain human mammary tumor cell lines.

The predicted human *erb*B-3 gene product is closely related to EGFR and *erb*B-2, which have been implicated as oncogenes in model systems and human neoplasia. The *erb*B-3 coding sequence was expressed in NIH/3T3 fibroblasts and its product was identified as a 180 kDa glycoprotein, $gp180^{erbB\text{-}3}$. Tunicamycin and pulse-chase experiments revealed that the mature protein was processed by N-linked glycosylation of a 145 kDa *erb*B-3 core polypeptide. The intrinsic catalytic function of $gp180^{erbB\text{-}3}$ was uncovered by its ability to autophosphorylate *in vitro*. Ligand-dependent signaling of its cytoplasmic domain was established employing transfectants which express a chimeric EGFR/*erb*B-3 protein, $gp180^{EGFR/erbB\text{-}3}$. EGF induced tyrosine phosphorylation of

the chimera and promoted soft agar colony formation of such transfectants. These findings, combined with the detection of constitutive tyrosine phosphorylation of gp180^{erbB-3} in 4 out of 12 human mammary tumor cell lines, implicates the activated erbB-3 product in the pathogenesis of some human malignancies.

Accordingly, in a principal embodiment, the present invention relates to a DNA segment having a nucleotide sequence that encodes an *erb*B-3 gene or a unique portion thereof. This portion of an *erb*B-3 gene includes at least about 12 to 14 nucleotides which are sufficient to allow formation of a stable duplex with a DNA or RNA segment having sequences complementary to those in this portion of an *erb*B-3 gene. Further, this unique portion of an *erb*B−3 gene, of course, has a sequence not present in an *erb*B or an *erb*B-2 gene. In other words, the sequence of this portion of an *erb*B-3 gene differs in at least one nucleotide from the sequence of any other DNA segment. In one embodiment, this DNA segment is exemplified by a human genomic DNA fragment that is produced by cleavage with the *Sac*I restriction enzyme, has a size of about 9 kbp, and is detectable by nucleic acid hybridization with a probe derived from the *v-erb*B gene only under reduced stringency hybridization conditions, as described in Example 1. By application of the nucleic acid hybridization and cloning methods described in the present disclosure, without undue experimentation, one of ordinary skill in the art of recombinant DNA is enabled to identify and isolate DNA fragments related to the present human DNA fragment comprising a nucleotide sequence that encodes at least a portion of a mammalian *erb*B-3 gene other than the human *erb*B-3 gene. Application of the genomic DNA fragment of the *erb*B-3 gene as a probe in hybridization methods also enables one of ordinary skill in the art to obtain an entire *erb*B-3 gene, by sequential isolation of overlapping fragments adjoining the present fragment, i.e., by an approach known in the art as chromosome walking.

The present disclosure describes the partial nucleotide sequence of the human genomic 9 kbp *Sac*I DNA fragment, within the region of homology

to the *v-erb*B gene; however, the methods in the present disclosure further enable the isolation and determination of the sequence of the entire 9 kbp human genomic DNA fragment according to the present invention. Accordingly, the present invention further relates to a DNA segment having the nucleotide sequence, or a unique portion thereof, of a human genomic DNA fragment that is produced by cleavage with the *Sac*I restriction enzyme, has a size of about 9 kbp, and is detectable by nucleic acid hybridization with a probe derived from the *v-erb*B gene only under reduced stringency hybridization conditions, as described in Example 1. By extension of the chromosome walking approach noted above, the present invention further enables one of ordinary skill in the art to determine the sequences of related DNA fragments comprising the complete human *erb*B-3 gene as well as *erb*B-3 genes of, for example, mammals other than human.

In the application of the present *Sac*I DNA fragment or any portion thereof as a probe for nucleic acid hybridization, the fragment is amplified, for example, by the *in vitro* polymerase chain reaction method (PCR; see U.S. Patent 4,683,202; U.S. Patent 4,683,195; and Saiki et al., 1985, *Science* 230:1350−54) or by standard methods of molecular cloning. For example, a clone of the human *erb*B-3 gene DNA segment according to the present invention is exemplified by a recombinant clone of a normal human thymus DNA fragment, herein designated as the E3-1 genomic clone, having the partial restriction enzyme map defined in Fig. 2 and the partial DNA sequence defined in Fig. 3 and SEQ ID NO:1 of the present application. Isolation and characterization of genomic clone E3-1 is described in Example 2, below.

Analysis of the nucleotide sequences of the human genomic DNA segment according to the present invention reveals that the nucleotide sequence encodes three open reading frames bordered by splice junction consensus sequences which define the boundaries between nontranslated intron sequences and the translated exons (shown in Fig. 2 and SEQ ID NO:1). The predicted amino acid sequences of the three exons (SEQ ID NOS:1 and 2) are highly

similar to three regions which are contiguous in the tyrosine kinase domains of *v-erb*B, as well as human EGFR and *erb*B-2 proteins. Moreover, the predicted amino acid sequences of this human genomic clone are included in a larger open reading frame in complementary DNA (cDNA) clones of an mRNA species that is detected by hybridization of a probe derived from the human genomic DNA clone.

Accordingly, the present invention also relates to a DNA segment having a nucleotide sequence of an *erb*B-3 gene in which that nucleotide sequence encodes the amino acid sequence of an *erb*B-3 protein or a unique portion thereof. In other words, the sequence of this portion of an *erb*B-3 amino acid sequence differs in at least one amino acid residue from the amino acid sequence encoded by any other DNA segment. This portion of an *erb*B-3 amino acid sequence includes at least about 4 to 6 amino acids which are sufficient to provide a binding site for an antibody specific for this portion of the *erb*B-3 polypeptide. Further, this unique portion of an *erb*B-3 amino acid sequence, of course, includes sequences not present in an *erb*B or an *erb*B-2 gene. In particular, the present invention relates to such a DNA segment for which this amino acid sequence or unique portion thereof is that of the polypeptide product of the human *erb*B-3 gene. This DNA segment is exemplified by the human genomic DNA clone E3-1, above, as well as by human cDNA cones designated E3−6, E3−8, E3−9, E3−11 and E3−16, which are described in Example 3 below. A preferred embodiment of this DNA segment that encodes the amino acid sequence of the entire polypeptide product of the human *erb*B-3 gene is human cDNA clone E3−16 having the nucleotide sequence defined in SEQ ID NO:3 and having the predicted amino acid sequence defined in SEQ ID NOS:3 and 4.

The DNA segments according to this invention are useful for detection of expression of *erb*B-3 genes in normal and tumor tissues, as described in Example 5 below. Therefore, in yet another aspect, the present invention relates to a bioassay for determining the amount of *erb*B-3 mRNA in a

biological sample comprising the steps of: i) contacting that biological sample with a nucleic acid isolate consisting essentially of a nucleotide sequence that encodes *erb*B-3 or a unique portion thereof under conditions such that a nucleic acid:RNA hybrid molecule, such as a DNA:RNA hybrid molecule, can be formed; and ii) determining the amount of hybrid molecule present, the amount of hybrid molecule indicating the amount of *erb*B-3 mRNA in the sample. Findings described in Example 5, below, indicate that increased *erb*B-3 expression, as detected by this method of this invention, plays a role in some human malignancies, as is the case for the EGFR (*erb*B) and *erb*B-2 genes.

Of course, it will be understood by one skilled in the art of genetic engineering that in relation to production of *erb*B-3 polypeptide products, the present invention also includes DNA segments having DNA sequences other than those in the present examples that also encode the amino acid sequence of the polypeptide product of an *erb*B-3 gene. For example, it is known that by reference to the universal genetic code, standard genetic engineering methods can be used to produce synthetic DNA segments having various sequences that encode any given amino acid sequence. Such synthetic DNA segments encoding at least a portion of the amino acid sequence of the polypeptide product of the human *erb*B-3 gene also fall within the scope of the present invention. Further, it is known that different individuals may have slightly different DNA sequences for any given human gene and, in some cases, such mutant or variant genes encode polypeptide products having amino acid sequences which differ among individuals without affecting the essential function of the polypeptide product. Still further, it is also known that many amino acid substitutions can be made in a polypeptide product by genetic engineering methods without affecting the essential function of that polypeptide. Accordingly, the present invention further relates to a DNA segment having a nucleotide sequence that encodes an amino acid sequence differing in at least one amino acid from the amino acid sequence of human *erb*B-3, or a unique portion thereof, and having greater overall similarity to the amino acid sequence of human *erb*B-3 than to that of any other polypeptide. The amino acid sequence of this DNA segment includes

at least about 4 to 6 amino acids which are sufficient to provide a binding site for an antibody specific for the portion of a polypeptide containing this sequence. In a preferred embodiment, this DNA segment encodes an amino acid sequence having substantially the function of the human *erb*B-3 polypeptide. As noted above, the predicted *erb*B-3 polypeptide is a 148 kd transmembrane polypeptide with structural features identifying it as a member of the *erb*B receptor family.

The similarity of the amino acid sequence of the present invention with that of an *erb*B-3 amino acid sequence is determined by the method of analysis defined by the sequence alignment and comparison algorithms described by Pearson and Lipman (Pearson, W. R. & Lipman, D. J., 1988, *Proc. Nat. Acad. Sci. U.S.A.* 85:2444−48). This comparison contemplates not only precise homology of amino acid sequences, but also substitutions of one residue for another which are known to occur frequently in families of evolutionarily related proteins sharing a conserved function.

The present invention further relates to a recombinant DNA molecule comprising a DNA segment of this invention and a vector. In yet another aspect, the present invention relates to a culture of cells transformed with a DNA segment according to this invention. These host cells transformed with DNAs of the invention include both higher eukaryotes, including animal, plant and insect cells, and lower eukaryotes, such as yeast cells, as well as prokaryotic hosts including bacterial cells such as those of *E. coli* and *Bacillus subtilis.* These aspects of the invention are exemplified by recombinant DNAs and cells described in Examples 2, 3 and 6, below.

One particular embodiment of this aspect of this invention comprises a cell, preferably a mammalian cell, transformed with a DNA of the invention, wherein the transforming DNA is capable of being expressed to produce the functional polypeptide of an *erb*B-3 gene. For example, mammalian cells (COS-1) transformed with the pSV2 gpt vector carrying the E3−16 cDNA

are prepared according to well-known methods, such as those described in U.S. Patent Application 07/308,302 of Matsui *et al.*, filed February 9, 1989; see also Pierce, J. H. *et al.*, 1988, *Science* 239:628–631; and Matsui, T., Heidaran, M., Miki, T., Popescu, N., La Rochelle, W., Kraus, M., Pierce, J. & Aaronson, S., 1989 *Science* 243:800–804). Briefly, cDNA expression plasmids are constructed by introducing the *erb*B-3-related cDNA encompassing all the nucleotides in the open reading frame into the pSV2 gpt vector into which the simian sarcoma virus long-terminal-repeat (LTR) had been engineered as the promotor, as previously described in detail. Transient expression of the *erb*B-3 gene in such recombinant vectors is achieved by transfection into COS-1 cells.

Stable expression of an *erb*B-3 gene can also be obtained with mammalian expression vectors such as the pZIPNEOSVX vector (Cepko, C. L., Roberts B. E. and Mulligan, R. C., 1984, *Cell* 37:1053–62). For example, a eukaryotic expression vector was engineered by cloning the full-length *erb*B-3 coding sequence derived from cDNA clone E3–16 into the *Bam*HI site of the pZIPNEOSVX vector DNA adapting the DNA fragments with synthetic oligonucleotides. NIH/3T3 cells were transfected with 1 μg of recombinant expression vector DNA (LTR*erb*B-3) and selected with the resistance marker antibiotic G418. To detect expression of *erb*B-3, polyclonal rabbit antiserum was raised against a synthetic peptide (such as amino acid (aa) positions 1191–1205 (SEQ ID NO:5); aa 1254-1268 (SEQ ID NO:6); aa 478-492 (SEQ ID NO:7); aa 1116-1130 (SEQ ID NO:8) and aa 1199-1213 (SEQ ID NO:9)). These peptide epitopes are located intracellularly within the predicted carboxyl terminus of the *erb*B-3 coding sequence with the exception of aa 478-492, which resides in the extracellular domain of the *erb*B-3 protein. For example, as shown in Fig. 7, immunoblotting analysis using antiserum raised against aa 1191-1205 led to detection of the *erb*B-3 protein (panel A). The specificity of *erb*B-3 protein detection was demonstrated by preincubating the antiserum with the homologous peptide (panel B). Moreover, the normal 180 kD *erb*B-3 protein was specifically detected with the polyclonal antiserum only in cell transfected with the recombinant *erb*B-3 expression vector, while control NIH3T3 cells that were not

transfected with the vector were negative. There was no cross-reactivity of the above-listed antisera with the related EGFR or *erb*B-2 proteins overexpressed in NIH/3T3 cells. The stably transfected NIH3T3 cells are useful as *erb*B-3 receptor protein sources for testing potential candidates for an *erb*B-3-specific ligand, analysis of the biological activity, as well as generation of monoclonal antibodies raised against the native *erb*B-3 protein. An *erb*B-3-specific ligand is identified by detection of autophosphorylation of the *erb*B-3 receptor protein, stimulation of DNA synthesis or induction of the transformed phenotype of the LTR*erb*B-3 transfected NIH3T3 cells.

Alternatively, other transformed cell systems are available for functional expression of receptors of the *erb*B receptor family, for example, a system based on the 32D cell line, a mouse hematopoietic cell line normally dependent on interleukin-3 (I1-3) for survival and proliferation. Recent studies have established that introduction of an expression vector for the EGFR in these cells leads to effective coupling with EGF mitogenic signal transduction pathways, thereby allowing a ligand of the EGFR to replace I1-3 in supporting survival and growth of the 32D cells. By employing the known methods described for the EGFR, for example (Pierce, J. H. *et al.*, 1988, *supra*), the E3−16 cDNA of the present invention is expressed to produce functional receptors in 32D cells which are then useful for examining the biological function of these *erb*B-3 receptors, for instance, the specificity of their ligand binding capacity and coupling capacities to secondary messenger systems. Thus, by so using gene expression methods described herein with the DNAs of the present invention, especially the preferred E3−16 cDNA clone, one of ordinary skill in the art, without undue experimentation, can construct cell systems which fall within the scope of this invention, for determining the mechanisms of *erb*B-3 regulatory processes. Accordingly, the present invention also relates to a bioassay for screening potential analogs of ligands of *erb*B-3 receptors for the ability to affect an activity mediated by *erb*B-3 receptors, comprising the steps of: i) contacting a molecule suspected of being a ligand with *erb*B-3 receptors produced by a cell that yields functional *erb*B-3 receptors; ii) determining the

amount of a biological activity mediated by those *erb*B-3 receptors; and iii) selecting those analogs which affect the biological activity mediated by the *erb*B-3 receptors. For example, a compound can be added to a cell having normal or low level *erb*B-3 phosphorylation. The amount of *erb*B-3 phosphorylation is then measured and compared to the level prior to adding the compound. The presence of increased activity can then be selected. Alternatively, a cell with high or constitutive *erb*B-3 phosphorylation can be used to screen for compounds which decrease activity. In addition, an *erb*B-3 ligand or analogs can be used in this system to screen for the amount of ligand which is necessary to promote or inhibit phosphorylation.

Various standard recombinant systems, such as those cited above as well as others known in the art, are suitable as well for production of large amounts of the novel *erb*B-3 receptor protein using methods of isolation for receptor proteins that are well known in the art. Therefore, the present invention also encompasses an isolated polypeptide having at least a portion of the amino acid sequence defined in Fig. 4 (SEQ ID NO:4), such as those polypeptides given by SEQ ID NOS:5-9.

The invention further presents results undertaken in an effort to identify and characterize the normal *erb*B-3 gene product (Examples 6-8). By analysis of an EGFR/*erb*B-3 chimeric receptor, this invention demonstrates that EGF-dependent activation of the *erb*B-3 catalytic domain results in a proliferative response in transfected NIH/3T3 cells. Further, the invention shows that some human mammary tumor cell lines exhibit a dramatic elevation of steady state *erb*B-3 tyrosine phosphorylation, implying functional *erb*B-3 activation in these tumor cells.

The identification of *erb*B-3 ligands is of great importance because, for instance, the availability of these ligands will facilitate the complete characterization of *erb*B-3 biological function as well as development of therapeutic strategies involving the ligands. In particular, the instant observation

of functional *erb*B-3 activation in mammary tumor cells at steady state raises the possibility that a role of *erb*B-3 in human tumors involves autocrine activation. That is, the simultaneous expression of the ligand by the tumor cell may constitutively activate *erb*B-3, leading to an uncontrolled proliferative growth response. Accordingly, this invention provides for the detection, purification and characterization of *erb*B-3 ligands, particularly *erb*B-3 ligands that are capable of either activating or down-regulating (blocking the activation of) the *erb*B-3 protein.

The ligand detection and purification method of this invention capitalizes on the *erb*B-3 expression and activation characteristics in certain cell lines as well as the common property of growth factor receptor tyrosine kinases to rapidly autophosphorylate on tyrosine residues in response to ligand triggering to detect activating or blocking ligand from source containing potential *erb*B-3 ligands, as described in Example 9. Therefore, in yet another aspect, the present invention relates to a method for detecting the presence of an *erb*B-3 ligand in a source containing a potential *erb*B-3 ligand, comprising the steps of a) contacting a first sample of cells from a cell line that expresses *erb*B-3 protein with the source containing a potential *erb*B-3 ligand for a time and under conditions sufficient to allow *erb*B-3 ligand contained in the source to bind to *erb*B-3 protein to form a triggered sample, wherein the cell line expresses *erb*B-3 protein having low level intrinsic tyrosine phosphorylation; b) contacting a second sample of cells from the cell line with a control medium (unconditioned serum free medium) for the time and under the conditions as given in step a) above to form a control sample; c) determining the level of *erb*B-3 activation in the triggered sample and in the control sample; and d) comparing the level of *erb*B-3 activation in the triggered sample with the level of *erb*B-3 activation in the control sample, wherein an increase in activation in the triggered sample over the control sample indicates the presence of an *erb*B-3 activating ligand in the source containing a potential *erb*B-3 ligand. Alternatively, chimeric receptors, as shown in Figure 11, can be utilized to screen for *erb*B-3 ligands. The *erb*B-3 activation can be ascertained by

measuring the level of *erb*B-3 tyrosine phosphorylation in the triggered sample and in the control sample (an increase in the level of *erb*B-3 tyrosine phosphorylation correlates with an increase in the level of *erb*B-3 protein activation); measuring the level of cell growth in the triggered sample and in the control sample (wherein an increase in the level of cell growth correlates with an increase in the level of *erb*B-3 activation) or measuring the level of DNA synthesis for the cells in the triggered sample and in the control sample (an increase in the level of DNA synthesis for the cells correlates with an increase in the level of *erb*B-3 activation).

Similarly, the presence of an *erb*B-3 blocking or inhibiting ligand in a source containing a potential *erb*B-3 ligand can be detected by a) contacting a first sample of a cell line that expresses *erb*B-3 protein with the source containing a potential *erb*B-3 ligand for a time and under conditions sufficient to allow *erb*B-3 ligand contained in the source to bind to *erb*B-3 protein to form a blocked sample, wherein the cell line expresses *erb*B-3 protein having high level intrinsic tyrosine phosphorylation; b) contacting a second sample of the cell line with a control medium for the time and under the conditions as given in step a) to form a control sample; c) determining the level of *erb*B-3 activation in the blocked sample and in the control sample; and d) comparing the level of *erb*B-3 activation in the blocked sample with the level of *erb*B-3 activation in the control sample, wherein a decrease in activation in the blocked sample over the control sample indicates the presence of an *erb*B-3 blocking ligand in the source containing a potential *erb*B-3 ligand. Alternatively, chimaric receptors, as shown in Figure 11, can be utilized to screen for *erb*B-3 blocking ligands.

In addition, the concentration of various ligands can be utilized to affect the *erb*B-3 activity. For example, a ligand which promotes *erb*B-3 activity at low concentrations can be administered or promoted to high concentrations which can inhibit *erb*B-3 activity.

This invention additionally provides a method of decreasing a biochemical or biological activity mediated by the *erb*B-3 receptor, comprising blocking the binding of an *erb*B-3 activating ligand with the *erb*B-3 receptor. The blocking can be accomplished by an antibody reactive with the ligand binding domain of the *erb*B-3 receptor or by an *erb*B-3 blocking ligand. Furthermore, a method of promoting a biochemical or biological activity mediated by the *erb*B-3 receptor, comprising contacting an *erb*B-3 activating ligand with the *erb*B-3 receptor is provided.

This invention also provides a method of detecting the overexpression of *erb*B-3 in a sample from a subject. The method comprises detecting the amount of *erb*B-3 in the sample and comparing the amount in the sample to the amount in an equivalent sample having normal expression, the presence of *erb*B-3 in a greater amount indicating overexpression of *erb*B-3. By "greater amount" is meant a statistically significant amount. Such amount depends on the conditions utilized and can readily be determined given the teachings set forth herein. Generally, a two-fold or greater increase would be predictive of overexpression. *erb*B-3 can be detected, for example, by detecting mRNA utilizing Northern hybridization, RNA dot blot, RNA slot blot, or *in situ* hybridization. *erb*B-3 can also be detected at the protein level utilizing, for example, Western blots, immunoprecipitation, immunohistochemistry, ELISA, and radioimmunoassay. Once overexpression is detected, the overexpression of *erb*B-3 can be correlated to a tumor. Such correlation can be used to diagnose a tumor or monitor the progression of a previously diagnosed tumor.

Also provided is a method of detecting the activation of *erb*B-3 in a test sample from a subject, comprising detecting the presence of phosphorylation of *erb*B-3, the presence of phosphorylation of *erb*B-3 indicating the presence of *erb*B-3 activation in the sample. This method can further comprise comparing the amount of *erb*B-3 phosphorylation in the test sample to the amount of *erb*B-3 phosphorylation in a sample from a normal subject and correlating an increase in phosphorylation in the test sample, with the presence

of a neoplastic condition in the subject. Such correlation can be used to diagnose a tumor or monitor the progression of a previously diagnosed tumor.

This invention further comprises a purified antibody specific for the human *erb*B-3 polypeptide having the amino acid sequence defined in Fig. 4 (SEQ ID NO:4) or the mature gp180$^{erbB\text{-}3}$ protein or a unique portion thereof, such as those polypeptides given by SEQ ID NOS:5-9. In this embodiment of the invention, the antibodies are monoclonal or polyclonal in origin, and are generated using *erb*B-3 receptor-related polypeptides or peptides from natural, recombinant or synthetic chemistry sources. The term "specific" refers to an *erb*B-3 antibody capable of binding or otherwise associating nonrandomly with an antigen of *erb*B-3 such that it does not cross react substantially with other antigens. These antibodies specifically bind to an *erb*B-3 protein which includes the sequence of such polypeptide. In other words, these antibodies bind substantially only to *erb*B-3 receptor proteins and not to *erb*B (EGFR) or *erb*B-2 proteins. Also, preferred antibodies of this invention bind to an *erb*B-3 protein when that protein is in its native (biologically active) conformation. For instance, MAb E-31 has been shown to detect the native *erb*B-3 protein.

Fragments of antibodies of this invention, such as Fab or F(ab)' fragments, which retain antigen binding activity and can be prepared by methods well known in the art, also fall within the scope of the present invention. Further, this invention comprises a pharmaceutical composition of the antibodies of this invention, or an active fragment thereof, which can be prepared using materials and methods for preparing pharmaceutical compositions for administration of polypeptides that are well known in the art and can be adapted readily for administration of the present antibodies without undue experimentation.

These antibodies and active fragments thereof, can be used, for example, for specific detection or purification of the novel *erb*B-3 receptor. Such antibodies could also be used in various methods known in the art for

targeting therapeutic drugs, including cytotoxic agents, to tissues with high levels of *erb*B-3 receptors, for example, in the treatment of appropriate tumors with conjugates of such antibodies and cell killing agents. Accordingly, the present invention further relates to a method for targeting a therapeutic drug to cells having high levels of *erb*B-3 receptors, comprising the steps of i) conjugating an antibody specific for an *erb*B-3 receptor, or an active fragment of that antibody, to the therapeutic drug; and ii) administering the resulting conjugate to an individual with cells having high levels of *erb*B-3 receptors in an effective amount and by an effective route such that the antibody is able to bind to the *erb*B-3 receptors on those cells.

The antibody of this invention is exemplified by rabbit antisera containing antibodies which specifically bind to *erb*B-3 protein. Such receptor specific antisera are raised to synthetic peptides representing a unique portion of the *erb*B-3 amino acid sequence, having six or more amino acids in sequences which are sufficient to provide a binding site for an antibody specific for this portion of the *erb*B-3 polypeptide. Further, this unique portion of an *erb*B-3 amino acid sequence, of course, includes sequences not present in an *erb*B or an *erb*B-2 amino acid sequence, as predicted by the respective cDNA sequences. The *erb*B-3 specific anti-peptide antibody of the present invention is exemplified by an anti-peptide antibody in polyclonal rabbit antiserum raised against any of the synthetic peptides given in SEQ ID NOS:5-9, which are derived from the predicted sequence of the *erb*B-3 polypeptide. The specific detection of *erb*B-3 polypeptide with antiserum raised against the peptide given in SEQ ID NO:5 is illustrated in mammalian cells transformed with an expression vector carrying a human *erb*B-3 cDNA (see Fig. 7). The antibody of this invention is further exemplified by *erb*B-3-specific monoclonal antibodies, such as the monoclonal antibody MAb E3-1, which was raised against the recombinantly expressed protein and is capable of detecting the native *erb*B-3 protein. MAb E3-1 specifically immunoprecipitated the mature 180 kDa *erb*B-3 protein from LTR-*erb*B-3 transfectants (Fig. 9A) and did not exhibit cross-reactivity with the EGFR or *erb*B-2 proteins.

Antibodies to peptides are prepared by chemically synthesizing the peptides, conjugating them to a carrier protein, and injecting the conjugated peptides into rabbits with complete Freund's adjuvant, according to standard methods of peptide immunization. For example, the peptide is synthesized by standard methods (Merrifield, R. B., 1963, *J. Amer. Soc.*, 85:2149) on a solid phase synthesizer. The crude peptide is purified by HPLC and conjugated to the carrier, keyhole limpet hemocyanin or bovine thyroglobulin, for example, by coupling the amino terminal cysteine to the carrier through a maleimido linkage according to well-known methods (e.g., Lerner R.A. et al., 1981, *Proc. Nat. Acad. Sci. USA*, 78:3403). In one standard method of peptide immunology, rabbits are immunized with 100 μg of the *erb*B-3 peptide-carrier conjugate (1 mg/ml) in an equal volume of complete Freund's adjuvant and then boosted at 10−14 day intervals with 100 μg of conjugated peptide in incomplete Freund's adjuvant. Additional boosts with similar doses at 10−14 day intervals are continued until anti-peptide antibody titer, as determined, for example, by routine ELISA assays, reaches a plateau.

The antibody can be labeled with a detectable moiety or attached to a solid support by methods known in the art to facilitate detection of an antibody/antigen complex. Such a detectable moiety will allow visual detection of a precipitate or a color change, visual detection by microscopy, or automated detection by spectrometry or radiometric measurement or the like. Examples of detectable moieties include fluorescein and rhodamine (for fluorescence microscopy), horseradish peroxidase (for either light microscopy or electron microscopy and biochemical detection), biotin-strepavidin (for light or electron microscopy) and alkaline phosphatase (for biochemical detection by color change). The detection methods and moieties used can be selected, for example, from the list above or other suitable examples by the standard criteria applied to such selections (Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1988).

Thus, by following the teachings of the present disclosure, including application of generally known immunological methods cited herein, one of ordinary skill in the art is able to obtain *erb*B-3-specific antibodies and use them in a variety of immunological assays, for example, for diagnostic detection of unusually high or low expression in normal or tumor tissues. Thus, the present invention also relates to a bioassay for detecting an *erb*B-3 antigen in a biological sample comprising the steps of: i) contacting that sample with an antibody of the present invention specific for an *erb*B-3 polypeptide, under conditions such that a specific complex of that antibody and that antigen can be formed; and ii) determining the amount of that antibody present in the form of those complexes.

The present invention may be understood more readily by reference to the following detailed description of specific embodiments and the Examples and Figures included therein.

DESCRIPTION OF THE FIGURES

Fig. 1. Detection of *v-erb*B-related DNA fragments in DNAs from normal human thymus (lane 1), human mammary tumor lines MDA-MB468 (lane 2), and SK-BR-3 (lane 3). Hybridization was conducted at reduced (panel A) or intermediate (panel B) stringency conditions. The arrow denotes a novel 9 kilobase pair (kbp) *erb*B-related restriction fragment distinct from those of the EGFR gene (*erb*B) and *erb*B-2;

Fig. 2. Genomic and cDNA cloning of *erb*B-3. The region of ($v-erb$B) homology within the genomic 9 kbp *Sac*I insert of λE3$-$1 was subcloned into the plasmid pUC (pE3$-$1) and subjected to nucleotide sequence analysis. The three predicted exons are depicted as solid boxes. *erb*B-3 cDNA clones were isolated from oligo dT-primed libraries of mRNAs from normal human placenta (shaded bars) and the breast tumor cell line MCF-7 (open bar). The entire nucleotide sequence was determined for both strands on *erb*B-3

complementary DNA from normal human placenta and upstream of the 5' *Xho*I site on pE3–16. The coding sequence is shown as a solid bar and splice junctions of the three characterized genomic exons are indicated by vertical white lines. Solid lines in the cDNA map represent untranslated sequences. Restriction sites: A=*Acc*I, Av=*Ava*I, B=*Bam*HI, Bg=*Egl*II, E=*Eco*RI, H=*Hind*III, K=*Kpn*I, M=*Mst*II, P=*Pst*I, S=*Sac*I, Sm=*Sma*I, Sp=*Spe*I;

Fig. 3. Nucleotide sequence of the region of *v-erb*B homology in the human *erb*B-3 gene derived from human genomic DNA clone E3–1, in the 1.5 kbp region from the *Eco*RI to the *Pst*I sites. This region contains three open reading frames bordered by splice junction consensus sequences (underlined). The predicted amino acid sequences of the three exons are shown in three letter code below the relevant DNA sequences;

Fig. 4. Comparison of the predicted amino acid sequence of the *erb*B-3 polypeptide with other receptor-like tyrosine kinases. The amino acid sequence is shown in single letter code and is numbered on the right. The putative extracellular domain (light shading) extends between the predicted signal sequence (solid box) at the amino-terminus and a single hydrophobic transmembrane region (solid box) within the polypeptide. The two cysteine clusters (Cys) in the extracellular domain and the predicted tyrosine kinase domain (TK) within the cytoplasmic portion of the polypeptide are outlined by dark shading. The putative ATP-binding site at the amino-terminus of the TK domain is circled. Potential autophosphorylation sites within the carboxyl-terminal domain (COOH) are indicated by asterisks. Potential N-linked glycosylation sites (↪) are marked above the amino acid sequence. The percentage of amino acid homology of *erb*B-3 in individual domains with *erb*B-2, EGFR, *met*, *eph*, insulin receptor (IR), and *fms* is listed below. Less than 16% identity is denoted by (-);

Fig. 5. Assignment of the genomic locus of *erb*B-3 was assigned to human chromosomal locus 12q13. A total of 142 grains were localized on the

400-band ideogram. As depicted in the diagram, specific labeling of chromosome 12 was observed, where 38 out of 51 grains were localized to band q13;

Fig. 6. Elevated *erb*B-3 transcript levels in human mammary tumor cell lines. A Northern blot containing 10 μg total cellular RNA from AB589 mammary epithelial cells (lane 1), as well as mammary tumor cell lines MDA-MB415 (lane 2) and MDA-MB453 (lane 3) was hybridized with an *erb*B-3 cDNA probe (panel A). Following signal decay the same blot was rehybridized with a human β-actin cDNA probe (Gunning, P., Ponte, P., Okayama, H., Engel, J., Blau, H. & Kedes, L., 1983, *Mol. Cell Biol.* 3:787-795);

Fig. 7. Expression of a human *erb*B-3 polypeptide in cells transformed by a cDNA segment as detected by an *erb*B-3-specific anti-peptide antiserum. Cellular lysates (100 μg of each sample) were electrophoresed and transferred to nitrocellulose membranes for analysis by Western blotting. Panel A. Detection of *erb*B-3 polypeptide with the antiserum. Panel B. Preincubation of the antiserum with homologous peptide. Antibody blocking indicates binding specificity. Lane 1: Selected cultures of NIH3T3 cells transfected with 1 μg LTR*erb*B-3 expression vector. Lane 2: control NIH3T3 cells;

Fig. 8. Characterization of gp180^{erbB-3} recombinantly expressed in NIH/3T3 cells. A: Immunoblot analysis of transfectants with *erb*B-3 peptide antisera MK4 and MK5 and peptide competition. B: Immunoprecipitation with MK5 antiserum of LTR-*erb*B-3 transfectants metabolically labeled for 2 h in the presence or absence of glycosylation inhibitor tunicamycin (1μg/ml). C: Pulse-chase analysis: LTR-*erb*B-3 transfectants were pulse labeled for 15 min with 0.5 mCi each of [^{35}S] methionine and [^{35}S] cysteine and immediately lysed (0) or chased with 100 μg/ml each of unlabeled methionine and cysteine for the indicated time periods. 1 x 10^7 TCA-precipitable counts were immunoprecipitated from total lysates using MK5 antiserum;

Fig. 9. Immunolocalization of gp180$^{erbB\text{-}3}$ on the surface of LTR-*erb*B-3 cells. A: Immunoprecipitation analysis of metabolically labeled LTR-*erb*B-3 transfectants with monoclonal antibody E3-1 and a non-immune control. B: Indirect immunofluorescence: Formalin-fixed LTR-*erb*B-3 transfectants were incubated with MAb E3-1 (upper left) or non-immune IgG (lower left) and stained with a fluorescein-conjugated secondary antibody (100x original magnification). Indirect immunofluorescence with MAb E3-1 of native LTR-*erb*B-3 cells (right panel; 1000x original magnification);

Fig. 10. Autophosphorylation *in vitro* and chronic tyrosine phosphorylation *in vivo* of gp180$^{erbB\text{-}3}$. LTR-*erb*B-3 or control lystaes were immunoprecipitated with *erb*B-3 monoclonal antibody (E3-1) or non-immune IgG (NI). Parallel immunoprecipitates were subjected either to immunoblot analysis with MK4 antiserum (A) or to an immunocomplex kinase assay in the presence of [^{32}P]-γATP (B). Tyrosine Phosphorylation *in vivo* was assayed by immunoprecipitation with monoclonal anti-P-Tyr antibodies followed by immunoblotting with MK4 antiserum (C);

Fig. 11. EGF-dependent tyrosine phosphorylation of an EGFR/*erb*B-3 chimeric receptor, gp180$^{EGFR/erbB\text{-}3}$. Serum-starved LTR-*erb*B-3, LTR-EGFR/*erb*B-3, and LTR-EGFR transfectants were triggered with 100 ng/ml EGF. Similar amounts of gp180$^{erbB\text{-}3}$, gp180$^{EGFR/erbB\text{-}3}$, and EGFR were immunoprecipitated with *erb*B-3 (E3-1) or EGFR (AB-1) monoclonal antibodies followed by immunoblot analysis with anti-P-Tyr antibodies or peptide antisera; and

Fig. 12. Activation of gp180$^{erbB\text{-}3}$ signaling function in human breast tumor cells. The *erb*B-3 protein was immunoprecipitated with MAb E3-1 from 1 mg total protein lysate and subjected to immunoblot analysis with *erb*B-3 peptide antiserum or phosphotyrosine antibodies as indicated.

DESCRIPTION OF SPECIFIC EMBODIMENTS

As used herein, the terms "polypeptide", "protein", "gene product", "antigen", "receptor", "receptor protein" and the like, when used in reference to *erb*B-3, encompass the *erb*B-3 amino acid functional sequence as given in SEQ ID NO:4, the mature *erb*B-3 glycoprotein, gp180$^{erbB\text{-}3}$, and these entities modified by other post-translational modifications, such as glycosylation or tyrosine phosphorylation. However, as is common in the art, the term "*erb*B-3 polypeptide" typically refers to the sequence as given in SEQ ID NO:4 while the remaining terms typically refer to gp180$^{erbB\text{-}3}$.

The identification of a third member of the *erb*B-EGF receptor family of membrane spanning tyrosine kinases and the cloning of its full length coding sequence is described the Examples herein. The presence of apparent structural domains resembling those of the EGF receptor suggests the existence of an extracellular binding site for a ligand. The structural relatedness of the extracellular domain of the *erb*B-3 receptor with that of the EGF receptor indicates that one or more of an increasing number of EGF-like ligands (Shoyab, M., Plowman, G. D., McDonald, V. L. Bradley, J. G. & Todaro, G. J., 1989, *Science* 243:1074–1076) interacts with the *erb*B-3 product. Accordingly, the *erb*B-3 gene is expected to play important roles in both normal and neoplastic processes, as is known for the EGFR and *erb*B-2 genes.

Despite extensive collinear homology with the EGF receptor and *erb*B-2, distinct regions within the predicted *erb*B-3 coding sequence revealed relatively higher degrees of divergence. For example, its carboxyl terminal domain failed to exhibit significant collinear identity scores with either *erb*B-2 or EGFR. The divergence at the carboxyl terminus also accounts for minor size differences among the three polypeptides of *erb*B-3, *erb*B-2, and EGFR, which possess estimated molecular weights of 148 kilodaltons (kd), 138 kd, and 131 kd, respectively. Within the tyrosine kinase domain, which represents the most conserved region of the predicted *erb*B-3 protein, a short stretch of 29 amino

acids closer to the carboxyl terminus than the ATP binding site differed from regions of the predicted *erb*B-2 and EGFR coding sequence in 28 and 25 positions, respectively. Such regions of higher divergence in their cytoplasmic domains are likely to confer different functional specificity to these closely related receptor-like molecules. Thus, mutations or other alterations in expression of the *erb*B-3 gene are likely to cause cancers or genetic disorders different from those associated with such defects in the *erb*B and *erb*B-2 genes.

Chromosomal mapping localized *erb*B-3 to human chromosome 12 at the q11−13 locus, whereas the related EGFR and *erb*B-2 genes are located at chromosomal sites 7p12−13 and 17p12−21.3, respectively. Thus, each gene appears to be localized to a region containing a different homeobox and a different collagen chain gene locus. Keratin type I and type II genes also map to regions of 12 and 17, respectively, consistent with the different localizations of *erb*B-3 and *erb*B-2, respectively. Thus, the DNA segments of the present invention represent novel probes to aid in genetic mapping of any heritable diseases which are associated with chromosomal aberrations in the vicinity of the 12q11−13 locus.

There is evidence for autocrine as well as paracrine effectors of normal cell proliferation. The former are factors that are produced by the same cells upon which they stimulate cell proliferation, whereas the latter factors are secreted by cells other than those that are affected by those factors. However, the inherent transforming potential of autocrine growth factors suggests that growth factors most commonly act on their target cell populations by a paracrine route. The present survey of *erb*B-3 gene expression indicates its normal expression in cells of epithelial and neuroectodermal derivation. Comparative analysis of the three *erb*B receptor-like genes in different cell types of epidermal tissue revealed that keratinocytes expressed all three genes. In contrast, melanocytes and stromal fibroblasts specifically lacked EGFR and *erb*B-3 transcripts, respectively. Thus, melanocytes and stromal fibroblasts may be sources of paracrine growth factors for EGFR and *erb*B-3 products, respectively,

that are expressed by the other cell types residing in close proximity in epidermal tissues.

Given that both *erb*B and *erb*B-2 have been causally implicated in human malignancy, the present findings (Example 5) that the *erb*B-3 transcript is overexpressed in a significant fraction of human mammary tumor cell lines indicates that this new member of the EGFR receptor family also plays an important role in some human malignancies.

Characterization of the human *erb*B-3 gene product, gp180$^{erbB\text{-}3}$, shows that it is a transmembrane glycoprotein exhibiting properties characteristic of a receptor-like tyrosine kinase. The recombinant human *erb*B-3 protein shared identical electrophoretic mobility with the natural *erb*B-3 product expressed in human breast tumor cell lines. Moreover, both recombinant and endogenously expressed gp180$^{erbB\text{-}3}$ were recognized by different antibodies directed against distinct epitopes, such as monoclonal (i.e., Mab E3-1) and peptide antibodies directed against epitopes in the extracellular and carboxyl-terminal domains. The 145 kDa *erb*B-3 polypeptide precursor conformed with predicted *erb*B-3 encoded protein following cleavage of its signal sequence. Finally, demonstration of its inherent signaling properties established functional integrity of recombinantly expressed gp180$^{erbB\text{-}3.}$

Although the *erb*B-3 tyrosine kinase domain shares greater than 60% amino acid identity with the EGFR and *erb*B-2 proteins, single amino acid substitutions differences in highly conserved residues shared among known tyrosine kinases raised a question as to whether *erb*B-3 harbors intrinsic catalytic activity involved in signal propagation instead of signal attenuation as has been postulated for certain receptor tyrosine kinase-like molecules (Chou et al., *Proc. Natl. Acad. Sci. USA* 88:4897 (1991)). Most notably, codon 834 within the tyrosine kinase domain predicts asparagine in *erb*B-3, while aspartate is present at this position in essentially all known protein kinases. Moreover, substitution of asparagine for aspartate in this position abolishes *c-kit* and *v-fps* tyrosine

kinase activity. The present characterization of the *erb*B-3 cytoplasmic domain demonstrates not only its catalytic function but also the ability to transduce a mitogenic signal as well. gp180^{erbB-3} demonstrated autokinase activity *in vitro* and, in some cell lines, tyrosine phosphorylation *in vivo*. Moreover, EGF-dependent activation of gp180$^{EGFR/erbB-3}$ was associated both with mitogenic signaling and *in vivo* tyrosine phosphorylation of the chimeric receptor. All of these findings imply that the *erb*B-3 protein represents a biologically active membrane spanning receptor capable of transducing a mitogenic signal in a ligand-dependent manner. Thus, the *erb*B-3 gene encodes a membrane spanning molecule possessing all the properties of a functional growth receptor.

Constitutive activation of *erb*B-3 catalytic activity was demonstrated in LTR-*erb*B-3 transfectants. These results raise the possibility that NIH/3T3 cells may express an *erb*B-3 ligand. If so, this putative ligand would unlikely interact with the EGFR, since overexpression of the latter in NIH/3T3 cells is not associated with its chronic tyrosine phosphorylation in the absence of exogenous EGF. This invention further established that EGF neither enhanced *in vivo* tyrosine phosphorylation of gp180^{erbB-3} nor elicited a mitogenic response in LTR-*erb*B-3 cells. Additional ligands of the EGF family, including TGFα, amphiregulin, and HB-EGF, have also failed to stimulate gp180^{erbB-3} tyrosine phosphorylation or DNA synthesis in LTR-*erb*B-3 cells. While a low affinity interaction of known EGF-related ligands for gp180^{erbB-3} cannot be excluded, these findings indicated that *erb*B-3 and EGFR proteins possess distinct ligand specificities. The ability to trigger the *erb*B-3 catalytic domain in the EGFR/*erb*B-3 chimeric molecule should make it possible to more readily identify its substrates as well as to compare them with those of its closely related family members.

Based upon this invention's demonstration that the *erb*B-3 protein is both catalytically active and can elicit a proliferative response in NIH/3T3 cells, the instant findings of its chronic activation in some human breast tumor cells suggest its contribution to the malignant phenotype in such tumors.

Analogous evidence has implicated overexpression associated with gene amplification of both EGFR and *erb*B-2 in a variety of tumors as well. In such tumors, there is precedence for activation of receptor kinase activity by mechanisms involving autocrine loops as well as genetic alterations affecting regulatory or coding sequences.

Both EGFR and *erb*B-2 genes have been implicated as oncogenes based upon demonstration of their overexpression and constitutive activation in various human tumors. The results of this invention argue strongly that the most recently identified family member, *erb*B-3, is activated in some human breast tumors. Overexpression of the *erb*B-3 protein did not invariably correlate with its chronic tyrosine phosphorylation. Hence, *erb*B-3 activation may involve autocrine stimulation or subtle genetic alterations. In addition to breast tumors, expression of the *erb*B-3 transcript has been observed in a wide range of human carcinomas, including colon, lung, kidney, pancreas, and skin. These findings prompt the search for evidence of *erb*B-3 activation as an oncogene in these other common human cancers.

Example 1. Identification of a Human DNA Fragment Related to the *erb*B-3 proto-oncogene Family

In an effort to detect novel *erb*B-related genes, human genomic DNA was cleaved with a variety of restriction endonucleases and subjected to Southern blot analysis with *v-erb*B as a probe. Normal mammary epithelial cells AB589 (Walen, K. H. & Stampfer, M. R., 1989, *Cancer. Genet. Cytogenet.* 37:249-261) and immortalized keratinocytes RHEK have been described previously (Rhim, J. S., Jay, G., Arnstein, P., Price, F.M., Sanford, K. K. & Aaronson, S. A., 1985, *Science* 227:1250−52). Normal human epidermal melanocytes (NHEM) and keratinocytes (NHEK) were obtained from Clonetics. Sources for human embryo fibroblasts (Rubin, J. S., Osada, H., Finch, P. W., Taylor, W. G., Rudikoff, S., & Aaronson, S. A., 1989, *Proc. Nat. Acad. Sci. USA* 86:802−806) or mammary tumor cell lines SK-BR-3, MDA-MB468, MDA-MB453, and MDA-MB415 (Kraus, M. H., Popescu, N. C., Amsbaugh, S. C. &

King, C. R., 1987, *EMBO. J.* 6:605–610) have been described. For nucleic acid RNA hybridization, DNA and RNA were transferred to nitrocellulose membranes as previously described (Kraus, M. H., *et al.*, 1987, *supra*). High stringency hybridization was conducted in 50% formamide and 5xSSC at 42°C. Filters were washed at 50°C in 0.1xSSC. Reduced stringency hybridization of DNA was carried out in 30% formamide followed by washes in 0.6xSSC, while intermediate stringency was achieved by hybridization in 40% formamide and washing in 0.25xSSC. For the specific results depicted in Fig. 1, DNAs were restricted with *Sac*I and hybridized with probe specific for an oncogenic viral form of the *erb*B gene, *v-erb*B, spanning from the upstream *Bam*HI site to the *Eco*RI site in the avian erythroblastosis proviral DNA (Vennstrom, B., Fanshier, L., Moscovici, C. & Bishop, J. M., 1980, *J. Virol.* 36:575–585).

Under reduced stringency hybridization, four *Sac*I restriction fragments were detected. Two were identified as EGFR gene fragments by their amplification in the mammary tumor cell line MDA-MB468 (Fig. 1A, lane 1,2) known to contain EGFR gene amplification and one as an *erb*B-2 specific gene fragment due to its increased signal intensity in another mammary tumor cell line, SK-BR-3, known to have *erb*B-2 amplified (Fig. 1A, lane 1,3). However, a single 9 kbp *Sac*I fragment exhibited equal signal intensities in DNAs from normal human thymus, SK-BR-3 and a line with high levels of EGFR, A431 (Fig. 1A). When the hybridization stringency was raised by 7°C, this fragment did not hybridize, whereas EGFR and *erb*B-2 specific restriction fragments were still detected with *v-erb*B as a probe (Fig. 1B). Taken together, these findings suggested the specific detection of a novel *v-erb*B-related DNA sequence within the 9 kbp *Sac*I fragment.

<u>Example 2.</u> <u>Cloning of the Human DNA Fragment Related to *erb*B</u>

For further characterization, a normal human genomic library was prepared from *Sac*I cleaved thymus DNA enriched for 8 to 12 kbp fragments. For convenience, bacteriophage λsep–lac5 was obtained from L. Prestidge and D. Hogness (Stanford University); many other cloning vectors derived from

phage λ or other genomes can be used for cloning this DNA fragment according to standard recombinant DNA methods that are well known in the art. Purified phage DNA was subjected to *cos*-end ligation, restriction with *Sac*I, and fractionation in a continuous 10−40% sucrose gradient. A genomic library was prepared by ligating *Sac*I restriction fragments of normal human thymus DNA in the molecular weight range of 8 kbp to 12 kbp (isolated by sucrose gradient sedimentation) with the purified phage arms. Ten recombinant clones detected by *v-erb*B under reduced stringency conditions did not hybridize with human EGFR or *erb*B-2 cDNA probes at high stringency. As shown in the restriction map of a representative clone with 9 kbp insert, the region of *v-erb*B homology was localized by hybridization analysis to a 1.5 kbp segment spanning from the *Eco*RI to the downstream *Pst*I site.

The nucleotide sequence of a portion of a clone of the novel human genomic DNA fragment related to *erb*B was determined for both DNA strands by the dideoxy chain termination method (Sanger, F., Nicklen, S. & Coulson, A. R., 1977, *Proc. Nat. Acad. Sci. USA*. 74:5463-67) using supercoiled plasmid DNA as template. The nucleotide sequence was assembled and translated using IntelliGenetics software. Amino acid sequence comparison was performed with the alignment program by Pearson and Lipman (Pearson, W. R. & Lipman, D. J., 1988, *supra*) as implemented on the computers of the NCI Advanced Scientific Computing Laboratory. Hydrophobic and hydrophilic regions in the predicted protein were identified according to Kyte and Doolittle (Kyte, J. & Doolittle, R. F., 1982, *J. Mol. Biol.* 157:105-132). Nucleotide sequence analysis revealed that the region of *v-erb*B homology in the 1.5 kbp segment from the *Eco*rI to the *Pst*I contained three open reading frames bordered by splice junction consensus sequences (Fig. 2). Computerized comparisons of the predicted amino acid sequence of these three open reading frames with other known proteins revealed the highest identity scores of 64% to 67% to three regions which are contiguous in the tyrosine kinase domains of *v−erb*B, as well as human EGFR and *erb*B-2 proteins. Furthermore, all splice junctions of the three characterized exons in the new gene were conserved with

*erb*B-2. Amino acid sequence homology to other known tyrosine kinases was significantly lower, ranging from 39% to 46%.

A single 6.2 kb specific mRNA was identified by Northern blot analysis of human epithelial cells using the 150 bp *Spe*I-*Acc*I exon-containing fragment as probe (Fig. 2). Under the stringent hybridization conditions employed, this robe detected neither the 5 kb *erb*B-2 mRNA nor the 6 kb and 10 kb EGFR mRNAs. All of these findings suggested that the present work has identified a new functional member of the *erb*B proto-oncogene family, which tentatively has been designated as *erb*B-3.

<u>Example 3. Cloning and Characterization of cDNAs for the mRNA of the Human *erb*B-3 Gene</u>

In an effort to characterize the entire *erb*B-3 coding sequence, overlapping cDNA clones were isolated from oligo dT-primed cDNA libraries from sources with known *erb*B-3 expression, utilizing gene-specific genomic exons or cDNA fragments as probes. In brief, an oligo dT-primed human placenta cDNA library in λgt11 was obtained from Clontech. MCF-7 cDNA was prepared by first strand synthesis from 5 μg poly A^+ RNA using an oligo dT containing linker-primer and Mo-MuLV reverse transcriptase, followed by second strand synthesis with DNA polymerase I, RNaseH, and subsequent T4 DNA polymerase treatment. Double-stranded cDNA was directionally cloned into the *Sfi*I site of λpCEV9 using specific linker-adapter oligonucleotides (Miki, T., Matusi, T., Heidaran, M. A. & Aaronson, S. A., 1989, *Gene* 83:137–146; see also, U.S. Application Ser. No. 07/386,053 of Miki et al., filed July 28, 1989). Following plaque purification, phage DNA inserts were subcloned into pUC-based plasmid vectors for further characterization. The clones were initially characterized by restriction analysis and hybridization to the mRNA, and were subsequently subjected to nucleotide sequence analysis. Clones designated pE3–6, pE3–8, pE–9, and pE3–11 carrying inserts with molecular weights ranging from 1.3 kbp to 4.3 kbp were isolated from a human placenta library, whereas the pE3–16 clone containing a 5 kbp insert was obtained by screening

the MCF-7 cDNA library with the upstream most coding sequence of pE3−11 as a probe. The clones pE3−8, pE3−9, pE3−11, and pE3−16 contained identical 3′ ends terminating in a poly A stretch (Fig. 2).

The complete coding sequence of *erb*B-3 was contained within a single long open reading frame of 4080 nucleotides extending from position 46 to an in-frame termination codon at position 4126. The most upstream ATG codon at position 100 was the likely initiation codon, as it was preceded by an in-frame stop codon at nucleotide position 43 and fulfilled the criteria of Kozak for an authentic initiation codon. The open reading frame comprised 1342 codons predicting a 148 kd polypeptide. Downstream from the termination codon, multiple stop codons were present in all frames. As shown in SEQ ID NO:4, the deduced amino acid sequence of the *erb*B-3 polypeptide predicted a transmembrane receptor tyrosine kinase most closely related to EGFR and *erb*B-2. A hydrophobic signal sequence of *erb*B-3 was predicted to comprise the 19 amino-terminal amino acid residues. Cleavage of this signal sequence between glycine at position 19 and serine at position 20 would generate a processed polypeptide of 1323 amino acids with an estimated molecular weight of 145 kd. A single hydrophobic membrane spanning domain encompassing 21 amino acids was identified within the coding sequence separating an extracellular domain of 624 amino acids from a cytoplasmic domain comprising 678 amino acids (SEQ ID NO:4).

The putative *erb*B-3 ligand-binding domain was 43% and 45% identical in amino acid residues with the predicted *erb*B-2 and EGFR protein, respectively. Within the extracellular domain, all 50 cysteine residues of the processed *erb*B-3 polypeptide were conserved and similarly spaced when compared to the EGFR and *erb*B-2. Forty-seven cysteine residues were organized in two clusters containing 22 and 25 cysteines respectively, a structural hallmark of this tyrosine kinase receptor subfamily (see, for example, Yamamoto, T., Ikawa, S., Akiyama, T., Semba, K., Nomura, N., Miyajima, N., Saito, T. and Toyoshima, K., 1986, *Nature* 319:230−234). Ten potential N-

linked glycosylation sites were localized within the *erb*B-3 extracellular domain. In comparison with the EGFR and *erb*B-2 proteins, five and two of these glycosylation sites were conserved, respectively. Among these, the site proximal to the transmembrane domain was conserved among all three proteins (SEQ ID NO:4).

Within the cytoplasmic domain, a core of 277 amino acids from position 702 through 978 revealed the most extensive homology with the tyrosine kinase domains of EGFR and *erb*B-2. In this region 60% or 62% of amino acid residues were identical and 90% or 89% were conserved, respectively. This stretch of amino acid homology coincides with the minimal catalytic domain of tyrosine kinases (Hanks, S. K., Quinn, A. M. & Hunter, T., 1988, *Science* 241:42–52).

There was significantly lower homology with other tyrosine kinases (Fig. 4). The consensus sequence for an ATP-binding site (GxGxxG, Hanks, S. K. *et al.*, 1988, *supra*) was identified at amino acid positions 716 through 721. This sequence as well as a lysine residue located 21 amino acid residues further toward the carboxyl terminus was conserved between the three *erb*B-related receptors. Taken together these findings defined the region between amino acid position 702 and 978 as the putative catalytic domain of the *erb*B-3 protein (SEQ ID NO:4).

The most divergent region of *erb*B-3 compared to either EGFR or *erb*B-2 was its carboxyl terminus comprising 364 amino acids. This region showed a high degree of hydrophilicity and the frequent occurrence of proline and tyrosine residues. Among these tyrosine residues, those at positions 1197, 1199, and 1262 matched closest with the consensus sequence for putative phosphorylation sites. The peptide sequence YEYMN (SEQ ID NO:12), encompassing tyrosine 1197 and 1199, was repeated at positions 1260–1264 and was at both locations surrounded by charged residues, providing an environment

of high local hydrophilicity. These observations render tyrosines 1197, 1199 and 1262 likely candidates for autophosphorylation sites of the *erb*B-3 protein.

Example 4. Chromosomal Mapping of the Human *erb*B-3 Gene.

The chromosomal location of the *erb*B-3 gene was determined by *in situ* hybridization (Popescu, N. C., King, C. R. & Kraus, M. H., 1989, *Genomics* 4:362–366) with a ^{3}H-labeled plasmid containing the amino-terminal *erb*B-3 coding sequence. A total of 110 human chromosome spreads was examined prior and subsequent to G banding for identification of individual chromosomes. A total of 142 grains was localized on a 400-band ideogram. Specific labeling of chromosome 12 was observed, where 38 out of 51 grains were localized to band q13 (Fig. 5). Thus, the genomic locus of *erb*B-3 was assigned to 12q13. In this region of chromosome 12, several genes have previously been mapped including the melanoma-associated antigen ME491, histone genes and the gene for lactalbumin. In addition, two proto-oncogenes, *int*-1 and *gli* are located in close proximity to *erb*B-3.

Example 5. *erb*B-3 Expression in Normal and Malignant Human Cells

To investigate its pattern of expression, a number of human tissues were surveyed for the *erb*B-3 transcript. The 6.2 kb *erb*B-3 specific mRNA was observed in term placenta, postnatal skin, stomach, lung, kidney, and brain, while it was not detectable in skin fibroblasts, skeletal muscle or lymphoid cells. Among the fetal tissues analyzed, the *erb*B-3 transcript was expressed in liver, kidney, and brain, but not in fetal heart or embryonic lung fibroblasts. These observations indicate the preferential expression of *erb*B-3 in epithelial tissues and brain.

*Erb*B-3 expression was also investigated in individual cell populations derived from normal human epithelial tissues including keratinocytes, glandular epithelial cells, melanocytes, and fibroblasts. For comparison levels of EGFR and *erb*B-2 transcripts were analyzed. As shown in Table 1, *erb*B-3 mRNA levels were relatively high in keratinocytes, comparable

with those of *erb*B-2 and EGFR in these cells. Lower, but similar expression levels of each transcript were detected in cells derived from glandular epithelium. These findings are consistent with growth regulatory roles of all three receptor-like molecules in squamous and glandular epithelium. Whereas *erb*B-2 and EGFR transcripts were also readily observed in normal fibroblasts, the same cells lacked detectable *erb*B-3 mRNA. In contrast, normal human melanocytes, which expressed both *erb*B-3 and *erb*B-2 at levels comparable with human keratinocytes, lacked detectable EGFR transcripts. Thus, the expression patterns of these receptor-like molecules were different in specialized cell populations derived from epidermal tissues.

Table 1: Normal expression pattern of human *erb*B gene family members

Cell Source of Transcripts	**Gene**	**Relative RNA levels**
Embryonic fibroblast (M426)	*erb*B-3	−
	*erb*B-2	+
	EGF-R	+
Skin fibroblast (501T)	*erb*B-3	−
	*erb*B-2	+
	EGF-R	+
Immortal keratinocyte (RHEK)	*erb*B-3	++
	*erb*B-2	++
	EGF-R	++
Primary keratinocyte (NHEK)	*erb*B-3	+
	*erb*B-2	+
	EGF-R	++
Glandular epithelium (AB589)	*erb*B-3	(+)
	*erb*B-2	(+)
	EGF-R	(+)
Melanocyte (NHEM)	*erb*B-3	++
	*erb*B-2	++
	EGF-R	−

Replicate Northern blots were hybridized with equal amounts (in cpm) of probes of similar specific activities for *erb*B-3, *erb*B-2, and EGFR, respectively. Relative signal intensities were estimated: − not detectable, (+) weakly positive, + positive, ++ strongly positive.

To search for evidence of *erb*B-3 involvement in the neoplastic process, *erb*B-3 mRNA levels in a series of human tumor cell lines were surveyed. The *erb*B-3 transcript was detected in 36 of 38 carcinomas of 2 of 12 sarcomas while 7 tumor cell lines of hematopoietic origin lacked measurable *erb*B-3 mRNA. Markedly elevated levels of a normal-sized transcript were observed in 6 out of 17 tumor cell lines derived from human mammary carcinomas. By Southern blot analysis, neither gross gene rearrangement nor amplification was detected in the cell lines. Fig. 6A shows the results of Northern blot analysis with control AB589 nonmalignant human mammary epithelial cells (lane 1) and two representative human mammary tumor lines, MDA-MB415 (lane 2) and MDA-MB453 (lane 3). Hybridization of the same filter with human β-actin probe (Fig. 6B) verified actual levels of mRNA in each lane. Densitometric scanning indicated that the *erb*B-3 transcript in each tumor cell line was elevated more than 100 fold above that of the control cell line. Thus, overexpression of this new member of the *erb*B family, as in the case of the EGFR and *erb*B-2 genes, is likely to play an important role in some human malignancies.

Example 6. Further Characterization of the normal *erb*B-3 gene product

The pZIPneo expression vector (Cepko et al, *Cell* 37:1053 (1984)) was modified by introduction of a unique Sal I cloning site. Following deletion of the Sal I site in the tetracyclin resistance gene, the synthetic oligonucleotides 5′-GATCTCGAGTCGAC-3′ (SEQ ID NO:10) and 5′-GATCGTCGACTCGA-3′ (SEQ ID NO:11) were annealed and ligated into the single Bam HI site to generate $pZIPneo_{Sal}$. The *erb*B-3 open reading frame including 7 nucleotides upstream of the initiation codon and the termination codon (nucleotides 93-4128) was linkered with Sal I ends, employing the polymerase chain reaction (PCR) and cloned into $pZIPneo_{Sal}$(LTR-*erb*B). Sense orientation and integrity of the open reading frame were confirmed by restriction analysis as well as nucleotide sequence analysis of cloning boundaries and PCR-amplified regions.

For structural and functional characterization of the *erb*B-3 gene product, the complete *erb*B-3 open reading-frame was inserted as given above into the modified ZIPneo vector, placing the cDNA under the transcriptional control of the Moloney murine leukemia virus long-terminal-repeat sequence (LTR-*erb*B-3). NIH/3T3 fibroblasts were transfected with LTR-*erb*B-3 or LTR-neo control DNA and cultured in the presence or absence of the selective drug G418. Under conditions in which efficient drug resistance (6×10^3 colonies/pmol) was conferred by LTR-*erb*B-3, no transformed foci were detectable. In contrast, LTR-*erb*B-2 or EGF-triggered LTR-EGFR induced morphological transformation of NIH-3T3 cells with efficiencies of around 1.2×10^4/pmol and 2.3×10^2/pmol, respectively.

To test for expression of the *erb*B-3 protein, polyclonal rabbit antisera, including MK4 and MK5, were developed against synthetic peptides. MK4 and MK5 were raised against peptides that encompass the residues given in SEQ ID NO:5 and SEQ ID NO:6, respectively, which are within the carboxyl terminus of the predicted *erb*B-3 product. For immunization, peptides were coupled to thyroglobulin using glutaraldehyde. Immunoblot analysis of lysates from marker-selected LTR-neo and LTR-*erb*B-3 transfectants revealed a major 180 kDa band only in LTR-*erb*B-3 cells. This band was independently recognized by both antisera (Fig. 8A). There was no cross-reactivity of either antiserum with the related EGFR or *erb*B-2 proteins overexpressed in NIH/3T3 cells. Immunoreactivity of either antiserum with the 180 kDa band in LTR-*erb*B-3 transfectants was competed by the antigenic peptide, while MK4 reactivity with a faint 125 kDa band was not affected by preincubation with peptide (Fig. 8A). These results established specificity of *erb*B-3 protein detection by both polyclonal antisera. By comparison, the 180 kDa *erb*B-3 protein migrated distinctly slower than the 170 kDa EGFR and slightly faster than the 185 kDa *erb*B-2 protein.

To characterize processing of the *erb*B-3 protein, we performed immunoprecipitation experiments with MK5 antiserum. Metabolic labeling of

LTR-*erb*B-3 transfectants in the presence or absence of tunicamycin demonstrated that the 145 kDa *erb*B-3 core polypeptide is modified by N-linked glycosylation (Fig. 8B). Pulse-chase analysis further indicated cotranslational processing, resulting in a predominant 170 kDa precursor protein in addition to faint *erb*B-3 specific bands of 150 kDa and 160 kDa, following 15 min of pulse-labeling (Fig. 8C). The mature 180 kDa *erb*B-3 protein appeared after 0.5 h of chase, and the majority was converted into gp180$^{erbB\text{-}3}$ by 1 h. By analysis of further time points, we estimate an approximate half-life of 2−3h (Fig. 8C). Thus, in NIH/3T3 cells, gp180$^{erbB\text{-}3}$ exhibits an apparently faster turn-over than the EGFR, for which a biosynthesis time of 3h and approximate half-life of 3−6 h has been reported.

For immunolocalization of the *erb*B-3 protein, *erb*B-3-specific monoclonal antibodies, including MAb E3−1, were raised against the recombinantly expressed protein. BALB/c mice were immunized with live LTR-*erb*B-3 cells. Somatic cell hybrids were prepared by fusion of immune splenocytes with murine non-secreting myeloma cells NS-1. Hybridoma supernatants were screened for differential immunoreactivity with LTR-*erb*B-3 but not LTR-neo transfectants by enzyme-linked immunosorbent assay (ELISA) using both live cells or cell extracts as antigen source. Positive hybridoma cell lines were cloned twice by limiting dilution and further characterized by immunoprecipitation and immunofluorescence analysis. One monoclonal antibody, MAb E3-1 (IgG2a isotype), specifically immunoprecipitated gp180$^{erbB\text{-}3}$ from LTR-*erb*B-3 transfectants (Fig. 9A) and did not exhibit cross-reactivity with the EGFR or *erb*B-2 proteins overexpressed in an NIH/3T3 cell background. Immunofluorescence analysis using a labeled second antibody revealed heterogeneous membrane immunostaining of formalin-fixed LTR-*erb*B-3 cells using MAb E3-1, but not with a non-specific immunoglobulin of matching isotype (Fig. 9B). MAb E3-1-specific membrane fluorescence of native LTR-*erb*B-3 cells (Fig. 9B) indicated that gp180$^{erbB\text{-}3}$ was expressed at the cell surface, as expected for a membrane-anchored protein.

To investigate its function, we next analyzed the *erb*B-3 protein for *in vitro* kinase activity. LTR-*erb*B-3 and control LTR-neo cell lysates were first immunoprecipitated with E3-1 followed by immunoblot analysis with MK4 antiserum (Fig. 10A). When the same immunoprecipitates were incubated in autokinase buffer containing $[^{32}P]-\gamma ATP$, a predominant 180 kDa phosphoprotein was labeled only in immunoprecipitates containing the *erb*B-3 protein (Fig. 10B). These findings indicated that $gp180^{erbB\text{-}3}$ possessed intrinsic protein kinase activity. To assess its enzymatic activity *in vivo*, LTR-*erb*B-3 lysates were subjected to immunoprecipitation with phosphotyrosine-specific monoclonal antibodies (anti-P-Tyr) followed by immunoblotting with MK4 antiserum. As shown in Figure 10C, the *erb*B-3 protein was recovered from anti-P-Tyr immunoprecipitates, and immunodetection was competed either by phenyl phosphate in the immunoprecipitation or the *erb*B-3 peptide in Western blot analysis. These findings indicated that recombinant $gp180^{erbB\text{-}3}$ expressed in NIH/3T3 cells was chronically phosphorylated on tyrosine residues.

The protein lysates were prepared in Staph A buffer containing the protease inhibitors phenylmethyl sulfonyl fluoride (1 mM) and aprotinin (10 μg/ml; Boehringer Mannheim). For the analysis of phospho-tyrosine proteins, the phosphatase inhibitors sodium orthovanadate (2 mM) and sodium pyrophosphate (10 mM) were added. Immunoblot analysis using peptide antisera was essentially conducted as previously reported. For the detection of phosphotyrosine proteins, membranes were blocked in PBS containing 5% BSA and immunostained with a mixture of monoclonal anti-P-Tyr antibodies (PY20 and PY69; ICN) diluted 1:500 in PBS containing 1% BSA. Filters were washed with PBS containing 0.05 % Tween20. Immunoprecipitation was conducted using gammabind G agarose (Pharmacia) to collect the immunocomplexes. The beads were coupled with goat anti-mouse-IgG second antibody (Boehringer Mannheim) in immunoprecipitations using *erb*B-3 or EGFR monoclonal antibodies. For *in vitro* kinase assays, 4 mg total lysates were precleared with gammabind G agarose. Following immunoprecipitation, washed immunocomplexes were equilibrated in autokinase buffer containing 40 mM

Hepes 7.5, 10 mM $MgCl_2$, and 0.05 % Triton. The immunocomplexes were subsequently divided for immunoblot analysis or immunocomplex kinase assay, respectively. Autokinase reactions were carried out in 40 μl autokinase buffer containing 20 Ci γATP (3000 ci/mmol) at 25° for 10 min and terminated by addition of SDS containing sample buffer.

Example 7. EGF-dependent mitogenic signaling by an EGFR/*erb* B-3 chimeric receptor

To explore *erb*B-3 signaling, a chimeric receptor, LTR-EGFR/*erb*B-3, containing the ligand-binding domain of the closely related EGF receptor (aa 1−682) and the intracellular portion of *erb*B-3 (aa 681−1342) was engineered. Linearized expression constructs (0.01-10 μg/plate) were transfected into NIH/3T3 cells by calcium phosphate precipitation using 40 μg of calf thymus DNA as carrier. Mass cultures expressing the recombinant proteins were obtained by selection with 750 μg/ml G418. Selected LTR-EGFR/*erb*B-3 transfectants were enriched for expression of the chimeric protein by preparative FACS sorting using EGFR monoclonal antibody AB-1 (Oncogene Sciences).

Transfection of NIH/3T3 cells with this construct did not result in detectable focus formation either in the presence or absence of EGF. To quantitate expression of the chimeric receptor, selected mass cultures were analyzed for EGF-binding in comparison to NIH/3T3 cells overexpressing the EGFR (LTR-EGFR). Scatchard analysis established around 5.7×10^5 EGF binding sites/cell for the LTR-EGFR/*erb*B-3 transfectant as compared to 2.5×10^6 binding sites/cell for LTR-EGFR transfectant. The LTR-EGFR/*erb*B-3 transfectant exhibited two populations of binding sites with affinities of 0.11 nM and 5 nM, respectively. The high-affinity sites were in the minority (2.3×10^4), and there were 5.5×10^5 low-affinity binding sites. Similar results were obtained with the wild-type EGFR in LTR-EGFR transfectants, which displayed 1.1×10^5 high affinity (0.13 nM) and 2.4×10^6 low affinity receptors (7 nM).

To investigate EGF responsiveness of *erb*B-3 enzymatic activity, the *in vivo* tyrosine phosphorylation of the chimeric receptor in the presence or absence of EGF was compared. This protein, as well as the EGFR and *erb*B-3 proteins from independent transfectants were enriched by immunoprecipitation and subjected to immunoblot analysis with either anti-P-Tyr or the appropriate specific antiserum. As shown in Figure 11, the steady state level of tyrosine phosphorylated gp180^{erbB-3} in NIH/3T3 cells was not altered upon EGF exposure (lane 1,2). The chimeric EGFR/*erb*B-3 receptor, which was expressed as a 180 kDa protein, gp180$^{EGFR/erbB-3}$ displayed low, but detectable level of tyrosine phosphorylation in serum-free medium (lane 3). However, EGF triggering of the chimera resulted in a substantial increase in tyrosine phosphorylation, demonstrating EGF-dependent activation of *erb*B-3 catalytic function (lane 4). The wild-type EGFR showed somewhat higher level of EGF-dependent tyrosine phosphorylation under the same conditions (lane 6). Of note, the relative level of gp180^{erbB-3} tyrosine phosphorylation was comparable to that of EGF-activated chimeric receptor expressed at a similar protein level, indicating constitutive activation of *erb*B-3 catalytic properties in LTR-*erb*B-3 transfectants.

Whether the *erb*B-3 catalytic domain was capable of transducing a mitogenic signal was then assessed. When the LTR-EGFR/*erb*B-3 transfectant was exposed to increasing EGF concentrations, there was a dose-dependent stimulation of DNA synthesis similar to that observed with EGFR overexpressing NIH-3T3 cells. Under the same conditions, neither LTR-neo nor LTR-*erb*B-3 transfectants showed a significant increase in DNA synthesis even at high EGF concentrations, consistent with previous observations. It should be noted that basal levels of DNA synthesis of the LTR-*erb*B-3 transfectant were 2–3 fold above those of the other transfectants, findings that were reproducible with several independent selected mass cultures.

The biological effects of activated *erb*B-3 catalytic function were assessed by testing the transfectants for anchorage-independent growth. To test anchorage-independent growth, cell suspensions were seeded at 10-fold serial

dilutions in semisolid agarose medium containing growth medium and 0.45% seaplaque agarose (FMC Corp.). Visible colonies comprising > 100 cells were scored at 14 days. EGF was added at a concentration of 20 ng/ml. Human mammary tumor cell lines were obtained from the American Type Culture Collection and propagated in Dulbecco's modified Eagle medium containing 10% fetal calf serum.

As shown in Table 2, LTR-neo transfectants failed to exhibit significant soft agar growth in the presence or absence of EGF. In contrast, EGF induced soft agar colony formation with both LTR-EGFR/*erb*B-3 and LTR-EGFR transfectants. The latter showed a larger colony number (Table 2) as well as colony size (data not shown). By comparison, the LTR-*erb*B-3 transfectant displayed EGF-independent colony formation with an efficiency similar to that of EGF-activated LTR-EGFR/*erb*B-3 transfectant (Table 2). All of these findings establish that ligand activation of a chimeric EGFR/*erb*B-3 receptor causes mitogenic signaling in NIH/3T3 cells and suggest that chronic tyrosine phosphorylation of *erb*B-3 in LTR-*erb*B-3 transfectants is associated with constitutive signaling in these cells.

Table 2: Anchorage-independent growth of NIH/3T3 transfectants

NIH3T3 transfectants	#colonies*/10^4 cells	
	-EGF	+EGF
LTR-neo	1 (± 1)	4 (± 2)
LTR-EGFR	2 (± 2)	206 (± 49)
LTR-EGFR/*erb*B-3	7 (± 3)	88 (± 16)
LTR-*erb*B-3	97 (± 20)	94 (± 29)

*mean (± standard error) of 3 independent assays

Example 8. Evidence for activated *erb*B-3 signaling function in human breast tumor cells

The availability of *erb*B-3 specific antibodies made it possible to explore expression and activity of gp180$^{erbB\text{-}3}$ in human tumor cells. Based upon our previous evidence for *erb*B-3 mRNA overexpression in certain breast cancer cell lines, we measured *erb*B-3 protein levels and tyrosine phosphorylation in such tumor lines, using the procedures given above. Following immunoprecipitation with Mab E3−1, immunoblot analysis with MK4 antiserum revealed the natural human *erb*B-3 product as a 180 kDa protein. The levels of *erb*B-3 protein varied markedly along the tumor lines analyzed, with highest expression in BT483, MDA-MB453, MDA-MB134, MDA-MB361, SK-BR-3, and MDA-MB468 (Fig. 12). The lowest levels were observed in BT20 and MDA-MB175 cell lines (Fig. 12), comparable with that expressed by nonmalignant 184B5 mammary epithelial cells (data not shown). Thus, *erb*B-3 protein expression varied by at least 20−30 fold among the lines tested, consistent with results of transcript analysis (data not shown).

Immunoblot analysis of the same immunoprecipitates with anti-P-Tyr antibodies revealed that tyrosine phosphorylation of the native *erb*B-3 product was undetectable in 8 of the tumor cell lines, including MDA-MB134, MDA-MB361, and MDA-MB468, which harbored increased *erb*B-3 levels. In contrast, *erb*B-3 protein expressed by 4 cell lines, including MDA-MB453, BT474, MDA-MB175, and SK-BR-3, demonstrated readily detectable chronic tyrosine phosphorylation (Fig. 12, lanes 5, 7, 9 and 11). In MDA-MB175, there was no significantly elevated level of *erb*B-3 protein. Thus, in 4 out of 12 breast tumor cells lines, the gp180$^{erbB\text{-}3}$ signaling function was activated at steady state. Whether chronic *erb*B-3 phosphorylation involves autocrine stimulation or subtle structural alterations, these findings provide evidence for constitutive gp180$^{erbB\text{-}3}$ activation in these human breast tumor cells.

Example 9. Identification, purification and characterization of *erb*B-3 ligands

As shown in Fig. 12, the gp180^{erbB-3} that is overexpressed in some human breast tumor cell lines can be either functionally activated or not, depending on the cell line. Further, in some other human breast tumor cell lines, the *erb*B-3 polypeptide is not overexpressed and, again, can be either activated or not activated. These differences, and the common property of growth factor receptor-like tyrosine kinases to rapidly autophosphorylate on tyrosine residues in response to ligand triggering, can be exploited to identify, isolate and characterize ligands, preferably specific ligands, that can activate or down-regulate *erb*B-3. The term "*erb*B-3 ligand" refers to a molecule that binds to the *erb*B-3 protein, particularly to the extracellular domain of the *erb*B-3 protein, and can activate ("*erb*B-3 activating ligand") or down-regulate ("*erb*B-3 blocking ligand") the biochemical and/or biological activity of the *erb*B-3 protein. Depending on the concentration of the ligand, a ligand can both activate and down-regulate activity.

A source containing a potential *erb*B-3 ligand, such as conditioned medium, body fluid extracts, cell extracts, tissue extracts or the like, with or without agents which can modify *erb*B-3 activity, can be screened for the presence of such a ligand by the ability of the solution, in the case of an activating ligand, to enhance *erb*B-3 phosphorylation. With respect to screening for an *erb*B-3 activating ligand, cells from a cell line whose expressed *erb*B-3 protein contains nonexistent or low level intrinsic tyrosine phosphorylation can be contacted with potential ligand sources or control medium for a time and under conditions sufficient to allow binding of an *erb*B-3 ligand, if present, to bind to *erb*B-3. Typically, if an *erb*B-3 ligand is present, binding will occur within a short time. Thus, the cells are exposed to potential ligand sources or control medium for, preferably, no longer than 30 minutes, most preferably, 10 minutes or less. Appropriate conditions to allow binding of the ligand can be determined by one skilled in the art, such as physiological conditions at 37°C or the conditions given in Fig. 5 of Holmes et al., *Science* 256:1205 (1992). *erb*B-3 modifying agents, if administered, can be present in a concentration between 10^{-2}

pM and 10^5 pM. The cell line employed can overexpress *erb*B-3, such as the mammary tumor cell lines MDA-MB415, MDA-MB134, MDA-MB468, BT483, MDA-MB361, MCF-7 and ZR-75-1, which express an increased amount of the *erb*B-3 protein with low level intrinsic tyrosine phosphorylation compared to protein amounts and activation levels for corresponding nonmalignant cells. One such potential activating ligand source can be derived from cell lines that not only overexpress *erb*B-3 but also exhibit high level intrinsic tyrosine phosphorylation, such as MDA-MB453, SK-BR-3, and BT474. In addition, any normal cell which does not overexpress *erb*B-3 can be utilized, e.g., fibroblasts.

Similarly, with respect to screening for an *erb*B-3 inhibitory down-regulating ligand, a cell line whose expressed *erb*B-3 protein contains high level intrinsic tyrosine phosphorylation can be exposed to potential ligand sources or control medium for a time and under conditions sufficient to allow binding of an *erb*B-3 ligand, if present, to bind to *erb*B-3. The cell line employed preferably expresses activated *erb*B-3, such as the mammary tumor cell lines MDA-MB453, SK-BR-3 and BT474. In addition, an activating ligand at higher concentration can be down-regulating. Such activity can be routinely screened given the teaching herein.

The triggering or blocking of *erb*B-3 activation can be detected by comparing the level of *erb*B-3 tyrosine phosphorylation in the cell line after exposure to the potential ligand source with the normal level, e.g., the level obtained after exposure to the control medium. For example, in a negative control the cells can be in serum free medium and for activating ligand the conditioned medium is from cell lines with increased *erb*B-3 that don't have phosphorylation. For instance, to measure *erb*B-3 specific tyrosine phosphorylation, potentially triggered (or blocked) cells and the control cells are lysed. Using procedures such as those discussed above, the *erb*B-3 protein is immunoprecipitated with an *erb*B-3 specific antibody, preferably a monoclonal such as MAb E3-1. The immunoprecipitates are divided and subjected to immunoblot analysis with either antiphosphotyrosine or *erb*B-3 antibodies. The

presence of an *erb*B-3 activating or blocking ligand can be monitored by a relative increase or decrease, respectively, of phosphotyrosine levels in comparison to the untriggered control. Any increase can be significant, especially a two-fold or greater increase. This ligand-detection system can be used repeatedly throughout the ligand purification procedures so as to monitor protein purification of the *erb*B-3 ligand to homogeneity.

Alternatively, following exposure of the cell lines to the potential ligand source as discussed above, detection of an *erb*B-3 activating ligand or blocking ligand can be accomplished by measurement of cell growth and/or mitogenic signals resulting from the activation or inhibition of *erb*B-3 catalytic activity, using, for example, the procedures given in Example 7 above. An increase in colony number or colony size and/or a dose-dependent increase of DNA synthesis for the cells exposed to the potential ligand relative to those exposed to the control medium correlates with the presence of an activating ligand in the potential ligand source. Conversely, respective decreases correlate with the presence of a blocking ligand in the potential ligand source.

Following the isolation and purification of the *erb*B-3 ligand, the identity of the ligand can be determined by protein identification methods known in the art, such as amino acid sequencing. Further, the *erb*B-3 ligand can be molecularly characterized. For instance, similar to the procedures outlined in Holmes et al., *Science* 256:1205 (1992), the nucleic acid sequence that corresponds to the ligand's amino acid sequence, or a partial amino acid sequence corresponding to a portion of the ligand, can be used to design degenerate oligonucleotide probes corresponding to the amino acid sequence or partial sequence. These degenerate oligonucleotides can be used to screen a cDNA library and generate a clone that encodes the precursor of the *erb*B-3 ligand. Following determination of the coding sequence, related coding sequences can be discovered by screening other libraries.

For purposes of completing the background description and present disclosure, each of the published articles, patents and patent applications heretofore identified in this specification are hereby incorporated by reference into the specification.

The foregoing invention has been described in some detail for purposes of clarity and understanding. It will also be obvious that various changes and combinations in form and detail can be made without departing from the scope of the invention.